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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 7/00, 7/01, 5/12
C12P 21/08, C07K 15/00
C12R 1/92

A1

(11) International Publication Number: WO 92/08785

(43) International Publication Date: 29 May 1992 (29.05.92)

(21) International Application Number: PCT/AU91/00519

(22) International Filing Date: 14 November 1991 (14.11.91)

(30) Priority data:
PK 3352 14 November 1990 (14.11.90) AU

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: DIAGNOSIS AND TREATMENT OF MULTIPLE SCLEROSIS

(57) Abstract

A morbillivirus, multiple sclerosis virus (MSV), is isolated from multiple sclerosis (MS) brain tissue. Antibodies which recognize or bind to MSV are used in diagnosis or treatment of MS. Serological diagnosis of MS comprises detection of circulating anti-MSV antibodies. Compositions and methods for producing an immune response against MS are also disclosed.

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DIAGNOSIS AND TREATMENT OF MULTIPLE SCLEROSIS.

This invention relates to the diagnosis of multiple sclerosis (MS) and in particular it relates to a method for diagnosis of MS by immunocytochemical staining of brain tissue, as well as to a method for serological diagnosis of MS. At present there is no single test available for the diagnosis of MS, and diagnosis is usually based on the results of several techniques including neurological examination, CAT scans, NMR imaging and CSF analysis, none of which is conclusive on its own.

The present invention also relates to the treatment of MS. In this respect, the invention provides antibodies to a virus which has been isolated from human MS brain tissue, and these antibodies may be used in direct therapy of MS patients. In addition, the present invention also provides a vaccine for use in producing an immune response to the virus isolated from MS brain tissue.

There are two main theories as to the cause of multiple sclerosis. One is that MS is an autoimmune-type disease and is based on the animal model of experimental allergic encephalomyelitis (EAE) and the fact that immunosuppression has a beneficial effect in some MS patients. Extensive studies on EAE over the past 50 years have however failed to explain the aetiology of MS but have helped to elucidate the role of the immune

system in the central nervous system (CNS). The second theory is that MS is a viral disease and is based on epidemiological studies and the demyelinating effect of some viruses, in particular the morbilliviruses such as measles (MV) and canine distemper (CDV) viruses.

Many studies have suggested that multiple sclerosis is a viral disease[1] and some emphasis has been placed on measles[2] and canine distemper[3] viruses. There are several reports of the isolation of viruses from tissues of MS patients[4] but none of these viruses has been established as the aetiological agent of this disease. A previous study[5], which used a peroxidase-labelled antibody against a paramyxovirus isolated from the central nervous system of cats, stained virus-like particles and nucleocapsid-like structures in phagocytic cells in MS plaques. The nucleocapsid-like structures have previously been referred to as "curved, linear profiles" (CLPs)[6]. The antibody against the feline virus has now been used in affinity chromatography to repeatedly isolate virus from the brains of 8 different confirmed cases of MS but not from 4 non-MS brains. Preliminary virological, immunocytochemical and polyacrylamide gel electrophoretic (PAGE) studies have indicated that this virus is related to MV and CDV, and this relationship has now been further confirmed by the ELISA-type assay. The association of this virus with demyelinating lesions in the CNS of domestic cats and MS patients indicates a possible causal relationship between these animals and the human disease[7].

A subclinical, primary demyelinating disease has been identified in the CNS of approximately 7% of cats examined[8,9] and a persistent, non-permissive paramyxovirus has been isolated from affected brain tissue[10]. Peroxidase-labelled antibodies raised against the cat virus stain virus-like particles and nucleocapsid-like

structures (CLPs) within MS plaques[5]. The staining reaction is blocked by pretreatment of MS brain tissue with sera from MS patients but not by pretreatment with non-MS human sera[5].

Based on this antigenic relationship, affinity chromatography has now been used to isolate CLPs and virus particles from human MS brain tissue. The antibodies to the cat virus, used in the immunocytochemical studies previously described, were absorbed to an affinity chromatography column containing one of several matrices and through this was passed homogenised brain tissue from MS and non-MS patients. CLPs and virus particles which conform to those of morbilliviruses were present in the eluate of the MS brain tissue but not in that of the non-MS tissue. The virus isolated from the MS tissue has been grown in culture and has been harvested using the same affinity chromatography technique. It is considered that the results obtained provide strong evidence that MS is due to persistent or latent viral infection. Such an aetiology is more consistent with the epidemiology of the disease than that of an autoimmune-type disease.

The virus isolated from MS brain tissue, hereinafter referred to as "multiple sclerosis virus" or "MSV", is a morbillivirus and is closely related to both the measles and canine distemper viruses. The isolated MSV grows *in vitro*, for example in CV 1 cells and cultures of oligodendrocytes, and can be further isolated from cultures of these cells.

In one aspect, the present invention provides MSV in isolated form, for example, as a culture of isolated MSV in CV 1 cells or in primary oligodendrocyte cultures. There is also provided a method for the preparation of MSV in isolated form.

In another aspect of the present invention there are provided antibodies which recognise or bind to the MSV morbillivirus, to an antigen of MSV or to an antigenic fragment thereof. These antibodies may be either polyclonal or monoclonal antibodies. In the case of monoclonal antibodies, the invention further extends to hybrid cell lines or hybridomas which produce such monoclonal antibodies.

The antibodies of this aspect of the invention may be produced by conventional techniques which are well known to persons skilled in the art using purified MSV isolated from MS brain tissue or from *in vitro* culture as described above. By way of example, hybrid cell lines or hybridomas producing monoclonal antibodies may be produced using the well known fusion technique first described in 1975 by Kohler and Milstein[11,12]. Polyclonal antibodies may be produced, for example, in laboratory animals such as rabbits, again by well known techniques[12].

Some monoclonal antibodies produced by the techniques outlined above have been shown to be very specific for MSV, whilst other monoclonal antibodies show varying degrees of cross-reactivity with MV and CDV.

In another aspect of the present invention there is provided a method for detection of MSV in a sample, such as a tissue sample, taken from a patient, which comprises contacting the sample with antibody which recognises or binds to MSV or an antigen of MSV or an antigenic fragment thereof, and detecting binding of said antibody to indicate the presence of MSV in the sample.

In this aspect, the polyclonal or monoclonal anti-MSV antibodies of this invention may be used in immunocytochemical staining of tissue from MS brains to confirm the diagnosis of the disease.

In another aspect of the present invention there is provided a method for the serological diagnosis of MS. This aspect of the invention is based on the observation that sera from MS patients contain circulating antibodies to MSV. Using immunocytochemical techniques, it has been shown that pretreatment of MS brain tissue with sera from MS patients, especially from patients who have recently had a relapse, blocks the immunocytochemical staining of the brain tissue previously described. This blocking demonstrates that the sera of MS patients contains antibodies to the virus.

Accordingly, in this aspect of the invention, there is provided a method for the detection of anti-MSV antibodies in a fluid sample taken from a patient, which comprises contacting said fluid sample with MSV or an antigen or antigenic fragment thereof, and detecting anti-MSV antibodies bound to said MSV or antigen or antigenic fragment thereof.

The fluid sample may be a blood or cerebrospinal fluid sample. Preferably, the sample is a blood sample, which may be whole blood or a derivative thereof, for example blood serum or blood plasma.

The diagnostic method of this aspect of the invention may utilise the well known principles of enzyme immunoassays or radio-immunoassays to detect the presence of any anti-MSV antibodies from the serum sample bound by the detecting antigen. The detecting antigen (MSV or an antigen or antigenic fragment thereof) may, for example, be immobilised on a solid support, and the presence of bound anti-MSV antibodies can be detected using appropriately labelled anti-human immunoglobulin antibody. Other alternatives will be well known to persons skilled in the art.

In this aspect of the invention, also, there is provided a diagnostic test kit for detection of anti-MSV antibodies in a fluid sample, which is characterised in that it includes MSV or an antigen or antigenic fragment thereof as detecting antigen, preferably immobilised on a solid support.

In yet another aspect of this invention which arises from the isolation and *in vitro* culture of MSV, there is provided a vaccine composition for stimulating an immune response against MSV in a human or animal patient, which comprises inactivated or attenuated MSV, or an antigen of MSV or antigenic fragment thereof, as the active immunogen.

In this aspect of the invention also, there is provided a method of producing an immune response against MSV in a human or animal patient, which comprises administration to said patient of an effective amount of an active immunogen comprising inactivated or attenuated MSV, or an antigen of MSV or antigenic fragment thereof.

If desired, the active immunogen may be coupled to a carrier molecule to improve its immunogenicity. Suitable carrier molecules may include, for example, haemocyanins such as keyhole limpet haemocyanin, bovine serum albumin or ovalbumin. In addition, the vaccine composition may optionally include an adjuvant. Known adjuvants for incorporation into animal and human vaccines include, for example, Freund's complete and incomplete adjuvants, alum, and the like.

Techniques are now well known for the production of inactivated or attenuated viruses, and, particularly in the case of MSV which is closely related to MV and CDV, attenuated vaccines have been developed against both of the latter viruses. Similarly, the preparation of

sub-unit virus vaccines is well known, based on the identification of antigens or antigenic fragments of the virus which are able to stimulate an immune response.

In the case of the MSV vaccine described herein, the vaccine may be produced for use in cats, similar to the canine distemper vaccine, and/or in humans, particularly human infants, similar to the measles vaccine.

In yet another aspect of the present invention there is provided a method of treatment of MS in a patient which comprises administering to the patient an effective amount of an antibody, preferably a monoclonal antibody, which recognises or binds to MSV, or an antigen of MSV or to an antigenic fragment thereof.

The antibody used in this aspect of the invention may be combined with a carrier or targeting molecule, for example carrier or targeting molecules such as intercellular adhesion molecules which assist the antibody to penetrate the blood brain barrier.

Further features of the present invention will be apparent from the detailed description in the following Examples.

EXAMPLE 1

Isolation of MSV from MS brain tissue and *in vitro* cultivation thereof in CV 1 cells.

Polyclonal antibodies to the persistent, non-permissive paramyxovirus isolated from cat brain tissue[10] were raised in rabbits. The immunoglobulins were purified from sera of inoculated animals, extensively adsorbed against cat liver powder, acetone dried Crandell feline kidney (CRFK) and Vero cells and concentrated to 5

mg of protein per ml. The prepared antibody was then absorbed to either Affi-Gel Protein A (Bio-Rad) or CNBr-activated Sepharose 4B (Pharmacia). One to two grams of frozen, autopsied brain tissue from 8 different, confirmed cases of MS and four non-MS cases were homogenised and passed through the affinity columns. The columns were washed with large volumes of Tris NaCl buffer prior to elution. The eluates were collected, drops were negatively stained with 3% phosphotungstic acid, pH 7.2, prior to examination in an electron microscope. Eluates obtained from the 12 brains were added to cultures of CV 1 cells.

Electron microscopy of the negatively stained eluates obtained after passage of MS brain tissue through columns of CNBr-activated Sepharose revealed pleomorphic, circular profiles with an average diameter of approximately 300 nm although some were considerably larger. The eluates obtained from the Affi-gel Protein A columns showed similar sized profiles but these lacked an external membrane and consisted of a tightly coiled, tubular-like, structure approximately 18 nm in diameter. The tubules had a thin translucent core and their walls had a "herring-bone" appearance consistent with a helical structure. Some fractions contained mostly tubular-like structures which were comparable to isolates of the nucleocapsid-like structures or CLPs[6]. Some eluants containing the virus-like particles were pelleted by centrifugation, embedded in agar and fixed and processed for electron microscopy. The pellets contained virions, up to 300 nm diameter, with internal tubular profiles, about 18 nm in diameter, sectioned in various planes. The viral isolation from MS brain tissue has been repeated more than 40 times.

After 5 passages, CV 1 cells infected with the eluates from the MS brains showed some focal cytopathic

effects (CPE) in the form of small syncytia and slight cytoplasmic vacuolation. Ultrastructural examination of these cultures revealed the presence of cytoplasmic inclusions which consisted of tubular structures approximately 18 nm in diameter. These inclusions were morphologically similar to those observed in the initial isolation of the feline paramyxovirus[10]. Virus particles, similar to those isolated from MS brains, were present in some cultures and were also isolated by use of the affinity chromatography technique.

Ultrastructural examination of the eluates obtained after passage of non-MS brain tissue through the columns did not reveal any viral particles. Cultures inoculated with these eluates did not show any CPE and neither cytoplasmic inclusions nor viral particles were seen when examined in the electron microscope. Furthermore, viral particles were not obtained from these cultures using affinity chromatography.

Polyacrylamide gel electrophoretic (PAGE) studies have shown that the peptides of the isolated virus are comparable with those of MV and CDV except for the absence of some membrane-spike proteins. This difference probably accounts for the lack of haemadsorption by infected CV 1 cultures of erythrocytes from chicken, cow, dog, guinea-pig, horse, human O, mouse, rabbit, rat and sheep. These observations were consistent with the properties of the feline paramyxovirus which exists in culture as a persistent non-permissive infection of CRFK and Vero cells[10].

The nucleocapsids and the virions isolated from MS brains show morphological similarities and some immunological cross-reactivity with MV and CDV. This, in addition to the initial PAGE studies and the formation of

small syncytia in tissue culture, suggests that the virus isolated from MS brains may be morbillivirus.

EXAMPLE 2

Production of Polyclonal Antibodies.

The nucleocaspid-like structures (CLPs) and viral particles obtained using the affinity chromatography procedure as described in Example 1 above were used in the production of polyclonal anti-MSV antibodies. 1.5 ml of the isolated MSV (5 µg/ml) was thoroughly mixed with 1.5 ml of complete Freund's adjuvant and administered by subcutaneous injection at several sites to a young adult rabbit. After 10 days, the rabbit was given a similar injection containing 1 ml of the viral isolate and 1 ml of incomplete Freund's adjuvant. This was repeated a further 5 times at 14 day intervals and then the animal was bled 8 days after the last inoculation. The rabbit was further inoculated three times with 1 ml of virus every 14 days over 42 days followed by bleeding 8 days after the third inoculation. This was repeated over a period of six months.

At the time of bleeding, the blood was allowed to clot and separate. The sample was then spun at 3000 rpm for 20 minutes and the clean serum was aspirated off and stored at -20°C. The immunoglobulin G was purified by addition of an equal volume of saturated ammonium sulphate $(NH_4)_2SO_4$ to the serum to precipitate the protein in the serum. The sample was then centrifuged at 3000 rpm for 30 minutes. The precipitate was retained and the supernatant was retreated with $(NH_4)_2SO_4$ and then recentrifuged.

The reserved precipitates were resuspended and further treated with an equal volume of 50/50 saturated $(NH_4)_2SO_4$/distilled water, before the solution was

centrifuged at 3000 rpm for 30 minutes. The precipitate was then dissolved in 0.01 M phosphate buffer solution.

The globulin proteins in the solution were then dialysed against several changes of phosphate buffered saline at 4°C overnight or for two days. The immunoglobulins were passed through a diethylaminoethyl cellulose (DEAEC) column of 50 cc capacity and the fractions collected. The optical densities of the fractions were determined using a spectrophotometer and the protein fractions pooled. The immunoglobulins were concentrated to about 5 mg/ml of protein and stored at -20°C.

For immunocytochemical studies, the polyclonal antibody was conjugated with horse-radish peroxidase according to the technique of Avrameas & Ternynck[13]. The cross-reactivity between MSV, MV and CDV has been demonstrated using an ELISA type assay, the results of which are shown in Figure 1.

EXAMPLE 3

Production of Monoclonal Antibodies.

Immunisation Protocol.

Six to eight week old Balb/C mice were immunised with an emulsion consisting of 0.1 ml of purified MSV (5 µg/ml) and 0.1 ml of complete Freund's adjuvant. The mice were injected in several sites subcutaneously. The booster immunisation, consisting of 0.1 ml of virus and 0.1 ml of incomplete Freund's adjuvant, was inoculated four weeks later subcutaneously in several places. A final injection of four times the original dosage of the virus in saline was given three to four days prior to fusion[14].

Myeloma Cell Line.

The myeloma cell line used was the Balb/C P3-NS1-Ag4-1 cell line (NS1). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 0.2 ml of penicillin/streptomycin (P/S), 120 µg/ml and 25 µg/ml respectively. The myeloma cells were incubated in 50 ml flasks at 37°C in a 7% CO_2 humidified incubator, and passaged through medium containing 8-azaquanine (2 µg/ml) to kill any revertant aminopteran resistant cells.

Myeloma cells were sub-cultured (split) every 24 hours, three days prior to fusion. Splitting of the cultures involved removing 50% of the medium, containing approximately 5 x 10^5 cells, and placing it into a new flask. Both flasks were then refilled to the original volume with fresh media so that each flask contained 2 to 3 x 10^5 cells per ml. The myeloma cells were never allowed to grow above a concentration of 8 x 10^5 cells per ml.

Perfusion of Spleen to obtain Lymphocytes.

Immunised mice were killed by CO_2 asphyxiation, the spleen removed aseptically and placed in a sterile petri dish containing 5 ml of culture medium. Spleen cells were washed out into the petri dish by injection of culture medium into the spleen capsule. A cell count was then performed.

Fusion.

The protocol used to perform fusions was adapted from that described by Galfre and Milstein[15]. Ten grams of polyethylene glycol (PEG) molecular weight 1500 (Sigma Chemicals), was liquefied and dispensed in 0.5 ml aliquots into pre-weighed bottles and autoclaved. The bottles were then weighed and the weight of PEG was calculated. Approximately 10^8 mouse spleen cells (those

obtained from 1 spleen) and 10^7 myeloma cells were mixed and placed in a sterile 50 ml conical-bottom tube. Serum free media was then added to produce a total volume of 50 ml. The mixture was then centrifuged at 400 g for five minutes and because the concentration of PEG is critical for fusion, all medium was removed to prevent dilution. The 0.5 ml aliquot of PEG was diluted to a final concentration of 40% (w/v) with serum free media and kept at approximately 40°C. The pellet was broken by gentle tapping and 1 ml of PEG added drop-wise over 1 minute. This was shaken for another 1 to 2 minutes. Then, 1 ml of serum free medium (pre-warmed to 37°C) was added over 1 minute. This was repeated and then performed twice more with the serum being added over 30 seconds. A further 7 ml of medium was added over 2 minutes, while being continuously shaken. Finally, 12 to 13 ml of medium was added and the cells were centrifuged at 400 g for 5 minutes. The supernatant was removed by suction and the pellet resuspended in 40 ml of HAT selection media containing 10^5 feeder cells per ml. [HAT medium: 50 ml of DMEM supplemented with 20% FCS, 0.4 ml penicillin/streptomycin, 0.05 ml Fungizone® (TAGO), hypoxanthine (136 µg/ml), aminopterin (0.19 µg/ml), thymidine (3.88 µg/ml), glutamine (2 mM) and pyruvate (1 mM).] The cells were then dispensed in 100 µl aliquots into 96-well culture plates (Linbro), and the plates were placed in an incubator at 37°C.

Post-Fusion Cell Maintenance.

Culture trays were maintained at 37°C in a 7% CO_2 humidified incubator. The plates were checked daily until Day 5 after fusion when large-scale cell death of spleen cells was expected and observed. Fresh HAT (50 ml per well) was added. At Day 7 to 14 after fusion, media (100 ml per well) was removed every 2 to 4 days and replaced with fresh HT media [HT media is the same as HAT but with the exclusion of aminopterin]. When the

majority of hybrids reached half confluence, 100 µl of supernatant was removed and screened for the appropriate antibodies. Positive clones were sub-cultured into new plates with the addition of spleen or peritoneal macrophage feeder cells.

Screening for Positive Hybrids.

Positive hybridomas were detected using enzyme-linked immunosorbent assay (ELISA). The Edmonston Strain of the measles virus (5 µg/ml) was diluted 1 in 40 in a solution containing 15 mM Na_2CO_3 and 33 mM $NaHCO_3$ solution (coating buffer) at pH 9.6 and 100 µl added to each well of a 96 well round bottom micro-titre plate (Disposable Products Australia). The level of the antigen used was optimised by the ELISA chequer board technique[14]. Plates were incubated for 18 hours at 4°C and then washed 3 times with a 0.5% solution of Tween 20 in phosphate buffered 0.15 M saline (PBS). Plates were then blocked with 10% FCS in PBS at room temperature for 2 hours, after which they were washed 3 times with PBS-Tween. Supernatant (100 µl) from the tissue culture plates was removed aseptically, added to the sensitised plates and incubated for 12 hours at 4°C. Wells were then washed and 100 µl of anti-mouse IgG conjugated with alkaline phosphatase (Tago, Inc., USA) diluted to 1 in 1000 was added. The plates were incubated for 2 hours at room temperature, and after washing, 100 µl of the enzyme substrate 4-nitrophenolphosphate (4-NPP) (Boehringer Mannheim, W.Germany), at 1 mg/ml in pH 9.8 10% diethanolamine supplemented with 0.5 mM $MgCl_2$ (substrate buffer) was added. Following a 30 minute incubation period at room temperature, the optical density was determined on a Titretek® Multistream microtitre plate reader using a 405 µm filter.

Preliminary Screening of CDV, MV and MSV.

Separate ELISA plates were sensitised with CDV vaccine (Websters), Edmonston measles virus and MSV according to the protocol previously described. Human polyclonal anti-measles antibodies, lot 25754, (Princess Margaret Hospital, Western Australia) were diluted with PBS to 1 in 320. The anti-CDV and anti-MSV antibodies were used at a concentration of 1 in 100, diluted with PBS. The enzyme conjugates used were anti-rabbit IgG alkaline phosphatase (TAGO) for CDV and MSV and anti-human alkaline phosphatase (TAGO) for the measles virus. After reconstitution, cell conjugates were used at a concentration of 1 in 1000 diluted with PBS. The ELISAs were carried out according to the protocol described above and the results are shown in Figure 1.

Screening Monoclonal Antibodies.

Six positive hybridomas were also screened against CDV, MV and MSV, as well as Newcastle Disease Virus (NDV) (10 µg/ml) and three feline viruses; Feline panleucopenia, Feline rhinotracheitis and Feline calcivirus [Websters 3 in 1 (living)] to check for any non-specificity. The feline viruses were used after being reconstituted with 1 ml of distilled water and subsequent dilution to 1 in 20 with PBS. The ELISAs were again carried out according to the protocol previously described. The results of this screen are shown in Figure 2.

EXAMPLE 4

Immunocytochemical Staining.

Polyclonal antibodies raised against MSV, as described in Example 2, has been used in immunocytochemical studies to identify MSV proteins within MS brain tissue, within CV1 cells and oligodendrocyte cultures infected with MSV and within

CRFK cells infected with the feline viral isolate. The staining technique used was essentially the same as that described by Cook *et.al.*[5] in the immunoperoxidase staining of MS brain tissue using polyclonal antibodies against the feline derived agent. The staining reaction product obtained with the polyclonal antibodies to MSV was associated with cytoplasmic inclusions mostly within phagocytic cells in MS plaques. Ultrastructural studies have confirmed that the stained inclusions were the tubular-like structures that have been called curved-linear profiles and which are in fact the nucleocapsid of the MS viral isolate. These cytoplasmic inclusions were also stained with horse-radish peroxidase-labelled monoclonal antibodies C1 and E1 (see Example 3 and Figure 2). These inclusions were not detected in normal white matter in MS brains nor in white matter from non-MS brains.

The immunocytochemical staining of cytoplasmic inclusions in MS plaques with horse-radish peroxidase-labelled polyclonal antibodies to MSV was blocked when the brain tissue was pre-incubated with sera from two MS patients who were experiencing a relapse. This blocking reaction was not as effective when serum was used from a patient in a remission phase of the disease. There was no reduction in the intensity of the staining reaction when the brain tissue was pre-incubated with sera from people who have no medical evidence of MS. Furthermore, horse-radish peroxidase-labelled immunoglobulin G (IgG) purified from the sera of two MS patients have been used to stain cytoplasmic inclusions within MS plaques. The results obtained are comparable to those seen when the labelled polyclonal antibodies to MSV are used. These results indicated that MS patients, especially those in which there is disease activity as indicated by clinical signs, have circulating antibodies to the MSV.

EXAMPLE 5

Oligodendrocyte culture system for MS virus

Primary cultures of oligodendrocytes have been grown and infected with the virus isolated from the brain tissue of two cases of MS. The results show that the virus has an affinity for oligodendroglia and does not infect astroglia. Furthermore, distinct cytopathic effects (CPE) are observed within 6 to 14 days post-infection. This compares with the 6 to 8 weeks that it takes to see obvious CPE when the virus is grown in CRFK, Vero or CV 1 cell lines. This culture system now allows a rapid assessment of the infectivity of the virus and will allow a comparison of strains of the virus for the detection of attenuated strains. The affinity of the virus for oligodendroglia provides further possible evidence that the demyelinating process in MS is due to a viral infection of these cells.

The oligodendrocyte culture system permits an evaluation of the effectiveness of monoclonal antibodies raised against the virus in inhibiting the growth of the virus.

One to two day-old Wistar rat pups were euthanased by cervical dislocation of CO_2 inhalation. Brain tissue was removed and dissociated using a modification of the technique used by Sarlieve *et.al.*[16]. Immediately following euthanasia, the pups were transferred to and immersed in 70% ethanol in a beaker and placed in a laminar flow cabinet. Brains were removed aseptically, washed in Dulbecco's Minimum Essential Medium (DMEM) with 10 Fetal calf sera (FCS) and transferred to a petri-dish containing a double layer of sterilised stainless-steel mesh (mesh size approx 100µm). Dissociation into fresh media (DMEM/10% FCS) was performed by repeatedly drawing and expelling the tissue through the mesh into and from a 20ml syringe. The resultant tissue suspension was

diluted with fresh media and seeded into 50ml/25cm Costar tissue culture flasks at 1-1.5 brains per flask with 10ml of media or onto poly-L-lysine-coated coverslips in 35 x 10mm petri-dishes at 0.5 brains per dish with 3ml of DMEM/10%FCS. The cultures were incubated at 37°C with 5% CO_2. Media was changed after the first 4-7 days and then weekly.

Inoculation of primary cultures with viral isolates from MS brain tissue.

Two isolates were obtained from brains A87/29 and MS-7 using the affinity chromatography technique previously described. These isolates were inoculated into 4 day-old primary rat glial cultures as follows. The media was removed from the cultures which were washed twice with fresh DMEM. The cultures were then inoculated with 100µl of virus suspension in 1ml of culture medium. Flasks were incubated for 2hr after which the original medium was returned to the culture without the removal of the virus suspension.

Immunocytochemistry

Fixation of cultures

Cultures were washed twice with phosphate buffered saline (PBS) to remove cell debris and fixed with a 5ml solution of 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer for 15 min. The fixative was removed and cultures were washed another two times with PBS prior to being stored in fresh PBS until stained.

Immunoperoxidase labelling of tissue cultures.

Cultures were washed twice with PBS and incubated for 2 min with ice-cold, 70% ethanol. This was followed by two washes with PBS over 15 min and incubation for 30 min with 10ml of 0.5% (v/v) H_2O_2 in Tris buffer. Flasks were washed with 40ml of Tris buffer and incubated for 435 min with 1ml of primary antibody (either HRP labelled

antibody raised against 2',3'-cyclic nucleotide 3'-phosphodiesterase [CNPase] or the MS viral isolate) at a dilution of 1:30 in Tris buffer or cultures were left in Tris buffer alone as controls. Following incubation, antiserum was removed by washing each culture three times over 10 min with 40ml of Tris buffer before the addition of 1ml of DAB solution and incubation, in the dark, for 15 min. After a further three washes in Tris buffer, the cultures were prepared for light or electron microscopy in a routine manner.

Isolation of virus particles from tissue culture media.

The medium was collected from cultures that had been infected for at least 11 days, i.e. these cultures had had at least one medium change. The pooled medium was centrifuged at 35,000 rpm for 3 hrs in a Beckman ultracentrifuge with a SW4 rotor. The pellet was resuspended with 0.5ml of phosphotungstic acid and a drop of this was placed on a Formvar grid for 5 min prior to drying and examination in an electron microscope.

Results

Primary rat oligodendroglial cultures.

Oligodendrocytes with a round cell antibody and 1 or 2 polar processes were identified after 3 days *in vitro*. Although these cells never reached confluency, they did show proliferation above the astrocytic bed layer. Other oligodendrocytes were comparable to those described as type I and type II oligodendrocytes by Kuhlmann-Krieg *et.al.*[17]. Type I cells had a triangular or ovoid shape with 2 or 3 processes and a flat cell body. Type II cells had a network of processes of various diameters and lengths surrounding the cell body. These oligodendrocytes tended to be above the astrocytic bedlayer. Astrocytes grew quickly to form a monolayer with cell to cell contact.

Immunocytochemical identification of oligodendrocytes.

The reaction product to the HRP labelled antiserum to CNPase, an enzyme specific to oligodendrocytes, was located in the cells described above as oligodendrocytes. There was no staining reaction with the underlying layer of astrocytes. The reaction product to CNPase was distributed throughout the cytoplasm of the oligodendrocytes and its location was the same irrespective of the age of the culture. There was, however, a slight decrease in the intensity of the staining in the longer-term cultures.

Controls.

There was very little, if any, DAB reaction product in control flasks.

Electron microscopy.

The above results were confirmed by electron microscopy. The antibody to CNPase gave a staining reaction which showed that this enzyme was in cells that had the ultrastructural characteristics of oligodendroglia and was located throughout the cytoplasm. Cells with features typical of astrocytes did not show positive staining.

Inoculation of cultures with the MS viral isolate.

6 days post-inoculation (10 days *in vitro*).

Some of the oligodendrocytes appeared to be enlarged and consisted of a central ring of condensed cytoplasm around which was a large membranous sheet-like structure. Some of these cells contained 2 to 3 nuclei. These cells were within the confluent layer of astrocytes rather than on top and there was a retraction of astrocytes around the oligodendrocytes.

9 to 14 days post-infection with the MS viral isolate.

In contrast to the 6 day post-infected cultures, these later cultures showed very obvious CPE. There were several large multinucleated glial cells within each culture. Some of these cells contained up to 30 nuclei, arranged as a ring around the periphery of the cytoplasm. These cells were positive for the labelled CNPase and contained cytoplasmic inclusions which were positive to the labelled antibody to the MS virus. The bedlayer of astrocytes had receded some considerable distance from the periphery of these cells suggesting that the multinucleated cells were producing soluble substances which may be cytokines.

Examination of the pellet obtained by ultra-centrifugation of the pooled media from the cultures infected with the MS virus showed that viral particles were present. This observation indicates that virus is being produced from the infected cells and that this is a more convenient and rapid method of obtaining large quantities of the virus. Although virus has been obtained from infected CRFK, Vero and CV 1 cells, the production of this virus has not been observed until 50 days or more post-infection. It should be noted that cultured oligodendroglia do not appear to be susceptible to the Edmonson strain of measles virus. Cultures infected with this virus did not show any CPE.

EXAMPLE 6

Detection of anti-MS antibodies in serum or cerebrospinal fluid.

Example 4 provides immunocytochemical evidence that there are circulating antibodies to MSV in the serum of MS patients. Purified MSV, an antigen or antigenic fragment derived from purified MSV, or a synthetic

polypeptide having a sequence substantially homologous (at least 75% homologous, preferably at least 90% homologous) with an antigen or antigenic fragment of MSV is used as the detecting antigen in an enzyme-linked immunosorbent assay (ELISA). Using known techniques, the detecting antigen is immobilised by being coated onto a solid support or carrier such as the surface of the wells of a microtitre plate, using coating buffer. After a washing step, the serum or cerebrospinal fluid sample is added to the wells. Following incubation, the wells are further washed, and an appropriate detecting antibody having an enzyme conjugated thereto (such as a goat anti-human IgG-horseradish peroxidase conjugate) is added. Enzyme activity bound to the solid support or carrier is detected using an appropriate enzyme substrate (such as 4-chloronaphthol or diaminobenzidine in the case of horseradish peroxidase) to indicate the presence of anti-MS antibodies in the sample.

EXAMPLE 7

Development of a vaccine to prevent MS.

The genus *Morbillivirus* includes measles and canine distemper as well as other viruses that have not been detected in Australia such as rinderpest virus, peste des petits ruminants, bovine encephalitis and phocine distemper virus. These viruses show a close antigenicity and there is considerable immuno-cross reactivity between some of the viral proteins. On the basis of immunological, morphological and virological studies, the MS virus is another member of this genus and has similar properties to measles and canine distemper viruses.

Live virus vaccines are considered to be better immunogens than inactive or killed viral vaccines. Effective measles and canine distemper vaccines are based on the use of a live virus and the production of these vaccines is well developed. The evidence that MSV is related to measles and canine distemper viruses, based on the immuno-cross reactivity described in Example 3, indicates that similar techniques can be used in the development of a vaccine using MSV.

The oligodendrocyte culture system (see Example 5) provides a means by which the infectivity of isolates or attenuated strains of MSV are assayed. Tissue culture infective doses (TCID) of the isolates or strains of the virus are evaluated and compared to identify an attenuated strain of the virus. The TCID of MS virus that has been grown in Vero and CV1 cells for several years, or virus that has been passaged through laboratory animals, are compared with the TCID of isolates obtaining directly from MS brain tissue to determine whether or not there is attenuation of the virus in the form of decreased infectivity of the oligodendroglial cells in culture.

The oligodendroglial culture system also permits an evaluation of the effectiveness of polyclonal and monoclonal antibodies raised against MSV on the virus in culture. The effectiveness of antibodies raised against attenuated forms of the virus is evaluated as well as the question of whether or not the attenuated forms produce a sufficient antibody response to act as an effective immunogen.

EXAMPLE 8

Development of a treatment for MS.

This invention provides forms of therapy of MS. The first of these is the use of an inoculum of a monoclonal antibody, such as C6 (Figure 2), which shows distinct specificity to MSV. The inoculation of varying quantities of this antibody will help to boost the patient's immune response to the virus. It is well recognised that not all MS lesions show an immune response in the form of a lymphocytic infiltrate of the tissue[18] and there is a lack of antibody producing plasma cells despite the presence of viral proteins in these lesions. However, a purified, monoclonal antibody may not penetrate the blood-brain barrier to provide an effective action against the virus even though this barrier is considered to be damaged due to oedema[18]. The antibody can then be conjugated with a carrier molecule, such as a cellular adhesion molecule or an oligodendrocyte specific molecule, which can target the antibody.

Treatment of MS may also take the form of an attenuated vaccine as described above or a sub-unit vaccine based on an antigen or antigenic fragment of MSV (or a synthetic polypeptide substantially homologous with an antigen or antigenic fragment of MSV), for example, the inoculation of a specific antigenic fragment of the virus such as the epitope to which the monoclonal antibody C6 (Figure 2) was produced. The advantage of this is that the immunogen will stimulate the immune system to produce B-lymphocytes which will produce large amounts of specific antibodies against MSV. The stimulation of the immune system in this manner will also increase the production of T-helper lymphocytes which will assist in the immune response in the lesions.

REFERENCES:

1. Liebowitz, S. in "Multiple Sclerosis" (eds. Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W.) (Chapman & Hall, London, 1983).

2. Norrby, E. Prog. Med.Virol. 24, 1-39 (1978).

3. Cook, S.D., Dowling, P.C. and Russell, W.C. J.Neurol. Sci. 41, 61-70 (1979).

4. ter Meulen, V. and Stephenson, J.R. in "Multiple Sclerosis" (eds. Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W.) (Chapman & Hall, London, 1983).

5. Cook, R.D., Flower, R.L.P. and Dutton, Neuropath. Appld. Neurobiol. 12, 63-79 (1986).

6. Prineas, J. Human Pathol. 6, 531-554 (1975).

7. Cook, R.D. Med.Hypoth. 7, 147-154, (1981).

8. Cook, R.D. Neuropath. Appld. Neurobiol. 5, 395-404, (1979).

9. Cook, R.D. and Wilcox, G.E. Neuropath. Appld. Neurobiol. 11, 361-367, (1985).

10. Wilcox, G.E. *et.al.* Vet. Microbiol. 9, 355-366, (1984).

11. Kohler, G. and Milstein, C. Nature, 256, 495-497 (1975).

12. Goding, J.W., "Monoclonal Antibodies: Principles and Practice" (Academic Press, 1986).

13. Avrameus, S. and Ternynck, T. Immunocytochemistry, 8, 1175-1179 (1971).

14. Campbell, A.M. "Monoclonal Antibody Technology" (Elsevier Publishers, Netherlands, 1984).

15. Galfre, G. and Milstein, C. Methods in Enzymology, 73, 3-46 (1981).

16. Sarlieve, L.L. *et.al.*. Brain Res., 189, 79-90 (1980).

17. Kuhlmann-Krieg, S. *et.al.*. Dev.Brain Res., 39, 269-280 (1988).

18. Adams, C.W.M. in "Multiple Sclerosis" (eds. Hallpike, J.F., Adams, C.W.M. and Tourtellotte, W.W.), (Chapman & Hall, London, 1983).

CLAIMS:

1. The morbillivirus, multiple sclerosis virus (MSV), in isolated form.

2. A culture of isolated MSV in CV 1 cells.

3. A culture of isolated MSV in primary oligodendrocytes.

4. A method for the preparation of MSV in isolated form, which comprises the step of contacting MSV-containing tissue with an immobilised antibody raised against feline paramyxovirus isolated from the central nervous system of cats, and recovering isolated MSV bound by said immobilised antibody.

5. A method for the preparation of MSV in isolated form, which comprises the step of contacting MSV-containing tissue with an immobilised antibody raised against MSV isolated from multiple sclerosis (MS) brain tissue, and recovering isolated MSV bound by said immobilised antibody.

6. A method of claim 4 or claim 5, wherein said MSV-containing tissue is MS brain tissue.

7. A method of claim 4 or claim 5, wherein after recovery said isolated MSV is cultured in CV 1 cells or primary oligodendrocyte cultures.

8. An antibody which recognises or binds to MSV, to an antigen of MSV, or to an antigenic fragment thereof.

9. An antibody of claim 8, which is a polyclonal antibody.

10. An antibody of claim 8, which is a monoclonal antibody.

11. A diagnostic reagent which comprises a labelled antibody of any one of claims 8 to 10.

12. A reagent of claim 11, wherein said labelled antibody comprises an enzyme-antibody conjugate.

13. A reagent of claim 12, wherein said conjugate comprises a horse-radish peroxidase-antibody conjugate.

14. A hybrid cell line or hybridoma which produces a monoclonal antibody which recognises or binds to MSV, to an antigen of MSV, or to an antigenic fragment thereof.

15. A method for the detection of MSV in a sample taken from a patient, which comprises contacting said sample with an antibody of any one of claims 8 to 10 or a diagnostic reagent of any one of claims 11 to 13, and detecting binding of said antibody or diagnostic reagent to indicate the presence of MSV in said sample.

16. A method of claim 15 wherein said sample is a brain or other tissue sample, and said detecting step comprises detecting binding of said antibody or diagnostic reagent to said sample to indicate the presence of MSV in said sample.

17. A method of claim 16, wherein said detecting step comprises immunocytochemical staining of said sample.

18. A method for the detection of anti-MSV antibodies in a fluid sample taken from a patient, which comprises contacting said fluid sample with MSV, an antigen or antigenic fragment thereof, or a synthetic polypeptide substantially homologous with an antigen or antigenic

fragment of MSV, and detecting anti-MSV antibodies bound to said MSV or antigen or antigenic fragment thereof.

19. A method of claim 18, wherein the detecting antigen comprises MSV or an antigen or antigenic fragment thereof immobilised on a solid support.

20. A method of claim 18 or claim 19, wherein bound anti-MSV antibodies are detected using labelled anti-human immunoglobulin antibody.

21. A method of any one of claims 18 to 20, wherein said fluid sample comprises a blood sample.

22. A method of any one of claims 18 to 20, wherein said fluid sample comprises a cerebrospinal fluid sample.

23. A test kit for the detection of anti-MSV antibodies in a fluid sample taken from a patient, characterised in that it includes MSV, an antigen or antigenic fragment thereof, or a synthetic polypeptide substantially homologous with an antigen or antigenic fragment of MSV, as detecting antigen.

24. A test kit of claim 23, wherein the detecting antigen comprises MSV or an antigen or antigenic fragment thereof immobilised on a solid support.

25. A composition for stimulating an immune response against MSV in a human or animal patient, which comprises inactivated or attenuated MSV, an antigen of MSV or antigenic fragment thereof, or a synthetic polypeptide substantially homologous with an antigen or antigenic fragment of MSV, as the active immunogen.

26. A composition of claim 25, wherein said active immunogen is coupled to a carrier molecule.

27. A composition of claim 26, wherein said carrier molecule is selected from keyhole limpet haemocyanin or another haemocyanin, bovine serum albumin or ovalbumin.

28. A composition of any one of claims 25 to 27, further comprising an adjuvant.

29. A composition of claim 28, wherein said adjuvant is selected from Freund's complete or incomplete adjuvants, or alum.

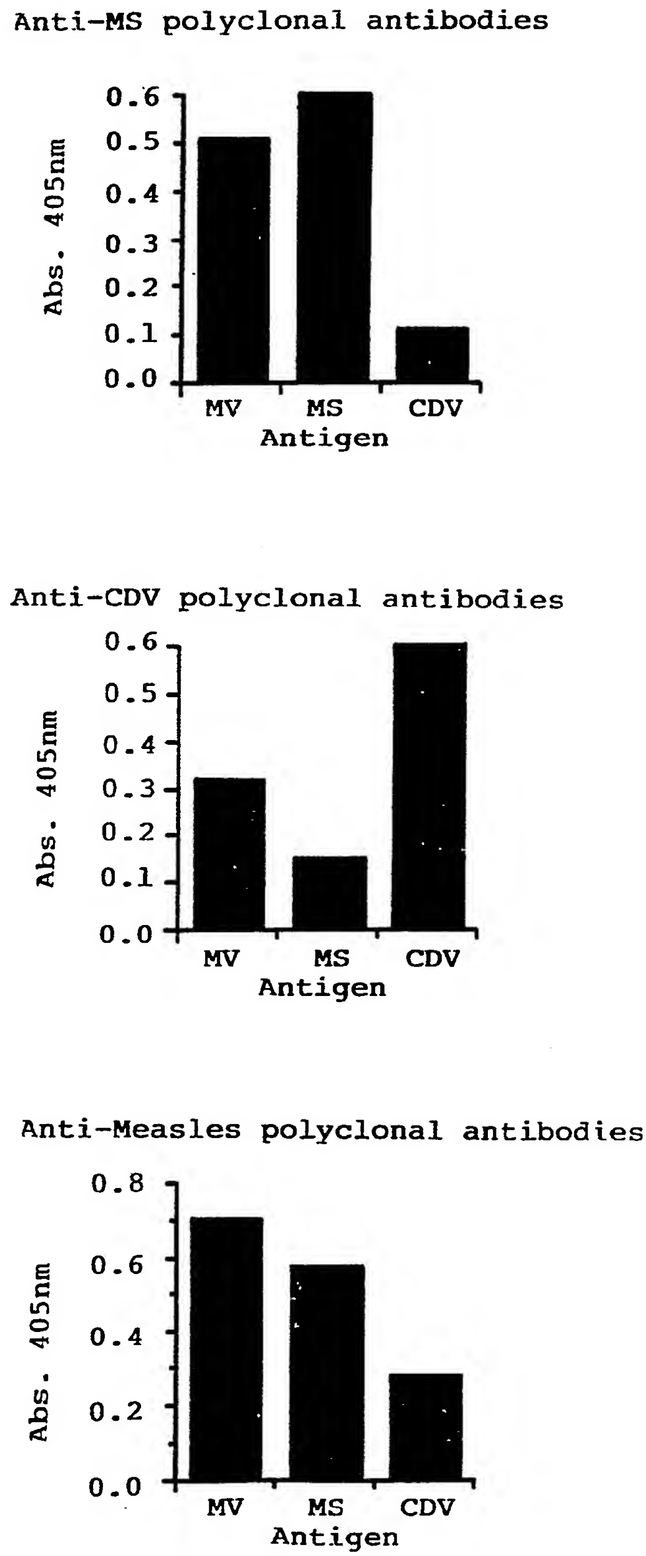
30. A method for producing an immune response against MSV in a human or animal patient, which comprises administration to said patient of an effective amount of a vaccine composition of any one of claims 25 to 29.

31. A method of treatment of MS in a patient, which comprises administering to said patient an effective amount of an antibody which recognises or binds to MSV, to an antigen of MSV, or to an antigenic fragment thereof.

32. A method of claim 31, wherein said antibody is a monoclonal antibody.

33. A method of claim 30 or claim 31, wherein said antibody is combined with a carrier or targeting molecule.

FIGURE 1


Anti-MS polyclonal antibodies
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0.5
0.4
0.3
0.2
0.1
0.0
MV
MS
CDV
Antigen
Anti-CDV polyclonal antibodies
Abs. 405nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MV
MS
CDV
Antigen
Anti-Measles polyclonal antibodies
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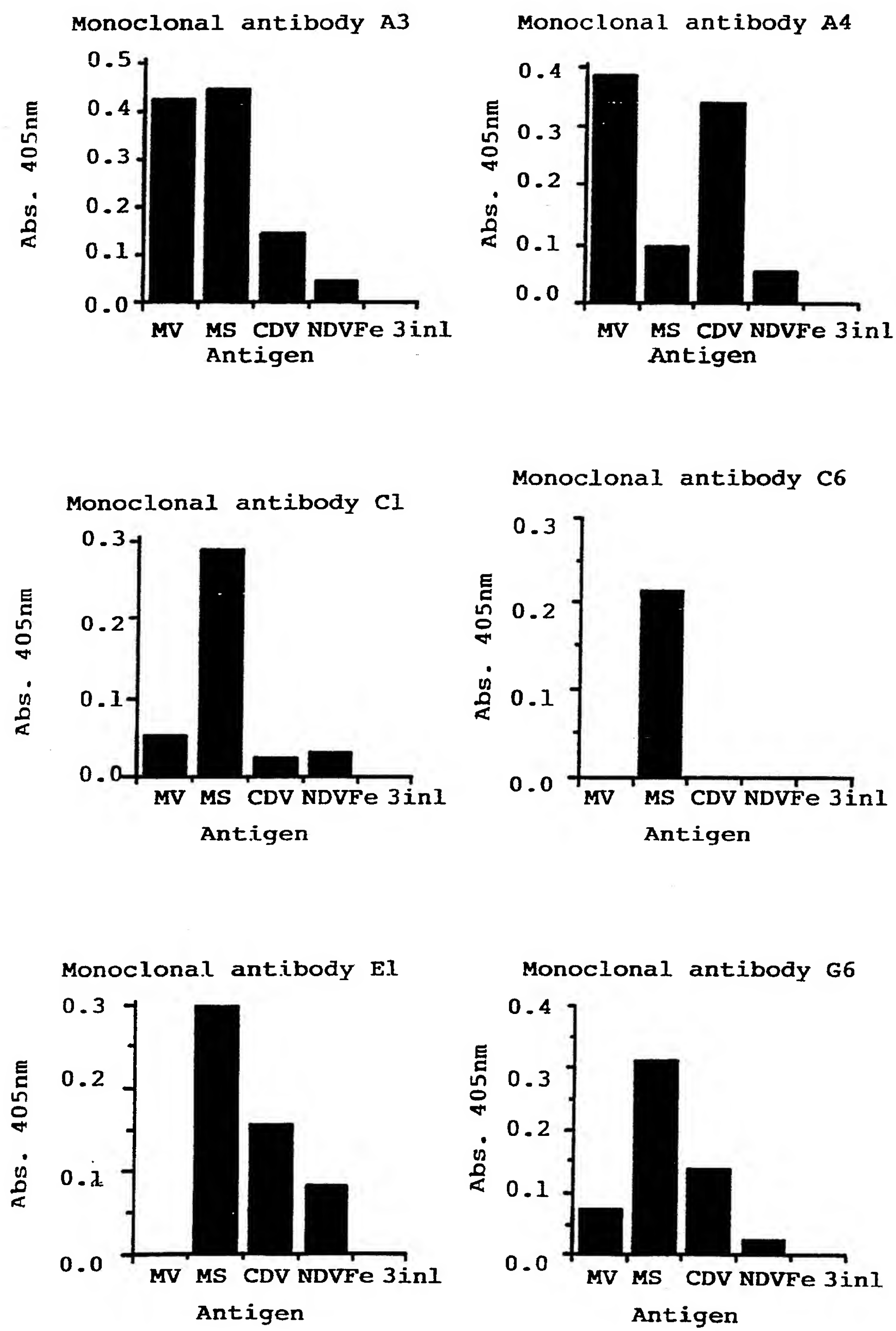
0.6
0.4
0.2
0.0
MV
MS
CDV
Antigen

FIGURE 2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0.1
0.2
0.3
0.4
0.5
MV MS CDV NDVFe 3inl
Antigen
Monoclonal antibody A4
Abs. 405nm
0.0
0.1
0.2
0.3
0.4
MV MS CDV NDVFe 3inl
Antigen
Monoclonal antibody C1
Abs. 405nm
0.0
0.1
0.2
0.3
MV MS CDV NDVFe 3inl
Antigen
Monoclonal antibody C6
Abs. 405nm
0.0
0.1
0.2
0.3
MV MS CDV NDVFe 3inl
Antigen
Monoclonal antibody E1
Abs. 405nm
0.0
0.1
0.2
0.3
MV MS CDV NDVFe 3inl
Antigen
Monoclonal antibody G6
Abs. 405nm
0.0
0.1
0.2
0.3
0.4
MV MS CDV NDVFe 3inl
Antigen

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC
Int. Cl.[5] C12N 7/00, 7/01, 5/12, C12P 21/08, C07K 15/00, C12R 1/92

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPAT Database: KEYWORDS: MULTIPLE SCLEROSIS VIRUS,MORBILLIVIRUS, MSV, MV Chemical Abstracts: KEYWORDS : as above

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: C12N 7/00, 7/01
BIOT: Biotechnology Abstracts Database: Keywords: as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
A	Immunology of Nervous System Infections, Progress in Brain Research Vol 59 (1983) pp 113-132. W.C. Russell. "Paramyxovirus and Morbillivirus Infections and their relationship to Neurolgical Disease (See whole document).	
A	Journal of Virology, Vol 52, No.3 (1984) pp 739-744 Melnick JL et al. "Characterisation of IM Virus which is frequently Isolated from Cerebrospinal fluid of Patients with Multiple Sclerosis and other Chronic Diseases of the central nervous system (see whole document).	

(continued)

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior t the international filing date but later than the priority date claimed
"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5 March 1992	13 MARCH 1992 (13.03.92)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	G. COLLINS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	AU,A, 28952/89 (CITY OF HOPE) 3 August 1989 (3.08.89) In particular page 3 line 20 - page 5 line 10.	
A	AU,B, 65043/86 (598209) (Koprowski H, Defreitas Ec, Gallo R.C.) 21 May 1987 (21.05.87) (see whole document)	

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers , because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.